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Award Number: W81XWH-05-1-0046

TITLE: Development of a Novel Vaccine Vector for Multiple CDC Category A Pathogens

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REPORT DATE: April 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
04-30-2008	Final	1 Apr 2007- 31 Mar 2008

4. TITLE AND SUBTITLE

Development of a Novel Vaccine Vector for Multiple CDC Category A Pathogens

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-05-1-0046

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Department of Defense
Department of Army
US Army Medical Research & Materiel Command
820 Chandler St.
Fort Detrick, MD 21702-5014

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Specific Aim 1 was to generate a panel of RhCMV/MPV vectors expressing MPV antigens A29L, A35R, M1R and B6R in either the wild type RhCMV vector, or in a vector lacking MHC immunomodulatory genes. To date, two vectors have been constructed and characterized, and we have subsequently selected one WT RhCMV vector (WTRhCMV/A35R) for immunogical characterization in rhesus macaques. Specific Aims 2 and 3 were to establish the pathobiology of WT MPV infection in RMs, and to monitor the immunological consequences of WT MPV infection. To date, eight RMs have been experimentally inoculated intrabronchially with MPV Zaire strain (MPVZ) to define a lethal dose by this route of infection and to characterize the virus/host interactions. Together, completion of these three specific aims will form the foundation for future studies designed to determine the efficacy of the RhCMV/MPV vectors at inducing a protective immune response to MPV challenge in RMs, and to identify viral determinants of pathogenesis.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRC
U	U	U	UU	13	**19b. TELEPHONE NUMBER** *(Include area code)*

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

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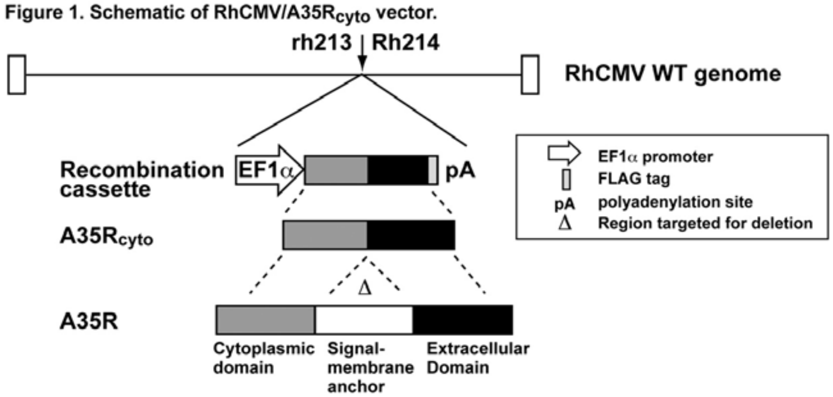
ABSTRACT:
The long-term goal of this project was to develop and evaluate human cytomegalovirus (HCMV) as an effective large capacity persistent vaccine vector to provide protective immunity for multiple bioterrorist agents and emerging diseases. To test this goal, we utilized the rhesus cytomegalovirus (RhCMV) vaccine vector system and the monkeypox virus (MPV)-rhesus macaque (RM) model. RhCMV is highly homologous to HCMV, and the MPV: RM model recapitulates all aspects of smallpox and monkeypox infection of humans. **Specific Aim 1** was to generate a panel of RhCMV/MPV vectors expressing MPV antigens A29L, A35R, M1R and B6R in either the wild type RhCMV vector, or in a vector lacking MHC immunomodulatory genes. To date, two vectors have been constructed and characterized, and we have subsequently selected one WT RhCMV vector (WTRhCMV/A35R) for immunogical characterization in rhesus macaques. **Specific Aims 2 and 3** were to establish the pathobiology of WT MPV infection in RMs, and to monitor the immunological consequences of WT MPV infection. To date, eight RMs have been experimentally inoculated intrabronchially with MPV Zaire strain (MPVZ). Two with 2 x 10^7 plaque forming units (PFU) and four with 2 x 10^5 PFU, to define a lethal dose by this route of infection and to characterize the virus/host interactions. Two additional RM were inoculated with 2 x 10^5 PFU of a recombinant MPVZ lacking the complement binding protein, MPVZ B14L ko. A summation of the studies is provided. Together, completion of these three specific aims will form the foundation for future studies designed to determine the efficacy of the RhCMV/MPV vectors at inducing a protective immune response to MPV challenge in RMs, and to identify viral determinants of pathogenesis.

INTRODUCTION:
The aims of the supported study were two-fold. One aspect was to ascertain the potential utility of human cytomegalovirus (HCMV) as a safe, potent, large capacity vaccine vector for CDC category A agents. HCMV possesses a number of unique characteristics, which potentially make HCMV an ideal vaccine vector to induce protective immunity against acute and chronic bioterrorist weapons. These characteristics include the lack of significant disease associated with infection, persistence of the virus for the lifespan of the host, an ability to re-infect HCMV seropositive individuals, and a capacity to induce a large memory T cell response. In the present study, we determined the utility of the CMV vector approach using the highly homologous rhesus cytomegalovirus (RhCMV) vaccine vector and infection of rhesus macaques (RM). The second aspect of the study was to develop and characterize the MPVZ infection of RM to better understand the pathogenesis associated with MPVZ infection and the host response to infection. The MPV: RM model is the only animal model that recapitulates all aspects of smallpox virus infection in humans and little is known about how the disease progresses and how the host responses to infection. Together, this study sufficiently demonstrates the utility of the CMV vaccine vector model as a means to protect the human population from biodefense related agents.

BODY:
Specific Aim 1: We will generate RhCMV-MPV vectors expressing the VV protective antigens that correlate to MPV-A29L, A35R, M1R and B6R.
SA1A: We will first construct a wild type (WT) RhCMV vector expressing the MPV protective antigens.
SA 1B: We will develop an RhCMV vector that lack the MHC immunomodulatory genes expressing the MPV protective antigens to determine if we can increase immunogenicity as well as increase vector space for other antigen expression cassettes.

Specific Aim 1A was completed in the previous funding period, and resulted in two series of RhCMV vectors each containing one of four individual MPVZ genes (A29L, A35R, M1R and B6R). As detailed in the previous report, the WTRhCMV/MPV vector expressing MPV antigen A35R (WTRhCMV/A35R) was selected for further immunological characterization in RMs. Immunization of RMs with WTRhCMV/A35R induced a significant CD4 and CD8 response against the target antigen (see below). Previous studies have shown that redirection of heterologous target antigens to the cytoplasmic compartment can augment the T cell response directed against the respective antigen (4). To determine whether a similar strategy would enhance the A35R-specific T cell response, a recombinant RhCMV vector that expresses a cytoplasmic-targeted A35R protein has been constructed (designated WTRhCMV/A35R$_{cyto}$; see schematic Figure 1). Based on sequence and biochemical analysis of its highly homologous vaccinia virus encoded orthologue, MPV A35R is predicted to be a type II protein with a hydrophobic region (amino acids 32-57) that serves as a combination signal sequence-membrane anchor (3). A cytoplasmic-targeted A35R protein (A35R$_{cyto}$) was constructed by removal of this signal-membrane anchor region. An A35R$_{cyto}$ recombination cassette comprised of the EF1α expressed FLAG-tagged A35R$_{cyto}$ gene was inserted at the desired position within the RhCMV BAC genome using linear recombination. Restriction enzyme digestion analysis and DNA sequencing of WTRhCMV/A35R$_{cyto}$ BAC clones showed the lack of aberrant genomic rearrangements, and confirmed sequence integrity of the A35R$_{cyto}$ open reading frame (data not shown). The WTRhCMV/A35R$_{cyto}$ virus has been reconstituted by transfection of BAC DNA into RhCMV-permissive RM fibroblasts. Transfected RM fibroblast cultures exhibited extensive cytopathic effect characteristic of RhCMV infection. Western analysis of cell lysates from these cultures showed expression of a FLAG epitope-tagged protein corresponding to the predicted size of A35R$_{cyto}$ (approx. 17kDa) (Figure 2). WTRhCMV/A35R$_{cyto}$ stocks are under preparation and will be analyzed for immunogenicity in RMs.

Figure 1. Schematic of RhCMV/A35R$_{cyto}$ vector.



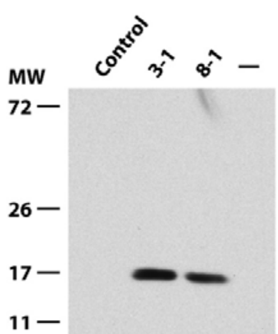


Figure 2. Western analysis of MPV protein expression from RhCMV/A35R$_{cyto}$ viruses. RhCMV/A35R$_{cyto}$ viruses (clones 3-1 and 8-1) were reconstituted by transfection of BAC DNA into RhCMV-permissive RM fibroblasts. Cells were harvested when reconstituted virus had spread through the culture, as observed by extensive virus-associated cytopathic effect. Expression of A35R$_{cyto}$ protein was determined using antibodies directed against the FLAG epitope tag. An epitope tag-reactive band of the size predicted for the tagged A35R$_{cyto}$ protein (approx. 17kDa) confirms expression of the A35R$_{cyto}$ protein in recombinant RhCMV/A35R$_{cyto}$ viruses. Control = control non-infected cellular lysate.

Specific Aim 2: We will establish the pathobiology of WT MPV infection of nonhuman primates

A. Experimental inoculation and clinical evaluation

We have performed several experimental animal infections with MPVZ and a deletion mutant to better understand the disease process and identify a lethal dose 50 (LD_{50}) when inoculated intrabronchially. From these infections, we determined that 2 x 10^7 PFU was a pathogenic dose as both animals infected with this dose succumbed to MPV-associated disease, similar to that reported (4).

Subsequent experimental inoculations were performed with 2 x 10^5 PFU, which initially resulted in the death of one of two animals due to MPV-associated disease. A second cohort of animals was inoculated again with 2 x 10^5 PFU and both animals became febrile, developed numerous pox lesions on their skin and oral cavity, and developed pneumonia. This cohort of animals resolved the infection and was euthanized at 39 days post-infection. From these experimental infections, we have determined that 2 x 10^5 PFU is sufficient to induce MPV-associated disease, which is characterized by numerous pox lesions and pneumonia. However, this dose is not a LD_{50}, as only one of four animals succumbed to the disease. As such, further animal infections are required to define this dose when delivered intrabronchially.

Table 1 summarizes the infections performed to date with MPVZ.

Table 1: Summary of experimental MPV infection

Animal	**Strain**	**Dose (PFU; plaque forming units)**	**Route** (ib; intrabronchial)	**Survival (days post-infection)**	**Clinical outcome**
19270	MPVZ	2 x 10^7	i.b.	10	Fever, few pox lesions, Euthanized due to pneumonia
20891	MPVZ	2 x 10^7	i.b.	10	Fever, few pox lesions, Euthanized due to pneumona
23225	MPVZ	2 x 10^5	i.b.	111	Fever, numerous pox lesions, monocytosis, pneumonia, animal survived
23983	MPVZ	2 x 10^5	i.b.	17	Fever, numerous pox lesions, monocytosis, euthanized due to pneumonia
23218	MPVZ	2 x 10^5	i.b.	39	Fever, numerous pox lesions, monocytosis, pneumonia, animal survived
23358	MPVZ	2 x 10^5	i.b.	39	Fever, numerous pox lesions, monocytosis, pneumonia, animal survived

As we reported in the last report, the second cohort of animals developed by day 7 pi and peak levels by day 12 pi (near 9-fold), which returned to pre-infection levels by day 25 pi. The increase in monocytes was further confirmed by fluorescent activated cell sorter (FACS) analysis using CD14 as a marker for monocytes.

B. Gross and histopathological examination

Animals euthanized due to complications due to MPV-associated disease exhibited widespread pox lesions in their oral cavity and on several internal organs. The lungs of all three animals were heavy and congested, and exhibited significant inflammation, consistent with

bronchopneumonia and appeared essentially as described by Zaucha *et al* (4). Additional finding were also consistent with those previously reported (4).

Animals euthanized after resolving MPV-infection did not harbor evidence of MPV-disease, except for fibrosis in the lungs, which were negative for virus isolation.

C. Virus isolation

Peripheral blood mononuclear cells (PBMCs) yielded recoverable virus by co-culture in the all animals. Interestingly viral loads coincided with the increase in circulating monocytes. Virus was also readily recovered from biopsied pox lesions, as well as from fluids recovered by bronchoalveolar lavage (BAL). Further evaluation of viral load in BAL and blood is being performed by real-time PCR.

Specific Aim 3: We will monitor the immunological consequences of WT MPV infection of non-human primates

SA3A: We will determine the antiviral CD4+ and CD8+ T cell responses to the MPV structural proteins, A29L and A35R, in MPV-infected RM.

SA3B: We will determine the antiviral antibody response mounted against the MPV structural proteins, A29L and A35R, in MPV-infected RM.

In our earlier report, we provided evidence that animals 23218 and 23358 both developed CD4+ and CD8+ T cell responses to MPV. Specifically, our data indicated that following MPV infection animals generate a strong T cell response dominated by the effector memory (EM) CD8 subset. Interestingly, EM CD8 T cells present in PBMC secrete higher levels of IFNγ in response to vaccinia stimulation than MPV stimulation. This might be indicative of immune evasion mechanisms by MPV that dampen the adaptive immune response in the periphery. This difference, however was not observed in the CD8 T cells obtained from the BAL where the IFNγ responses generated after MPV or VV infection were equivalent (Fig3 A). This discrepancy could be due to the different antigen presenting cell populations present in the lung versus peripheral blood. Alternatively this difference could be due to the difference in activation status of the T cells recruited to the site of infection (lung) versus those that circulate in peripheral blood.

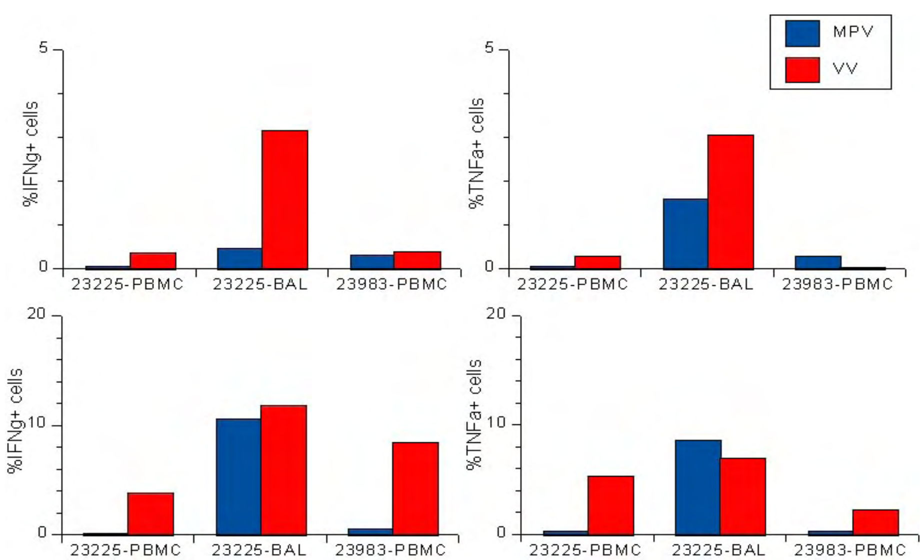


Figure 3 (A) This figure shows a comparison of representative CD8 T cell responses generated in both PBMC and BAL. The data is divided amongst the two major memory subsets: central memory (CM) and effector memory (EM). A higher percentage of cells with the CD8 EM subset secrete IFNg and TNFa after stimulation with VV and MPV especially in BAL. This is expected since the lung was the site of infection.

Peripheral CD4 T cells generated a much smaller response than their CD8 counterpart and lung-resident CD4 T cells did not respond to either VV or MPV stimulation (Fig 3B). There is a possibility that CD4 T cells might act through a different mechanism than IFNγ or TNFα secretion and we therefore were unable to detect antigen-specific CD4 T cells in using our current staining scheme. To address this possibility, we are currently investigating whether CD4+ cells are alternatively secreting IL-2 and/or IL-4.

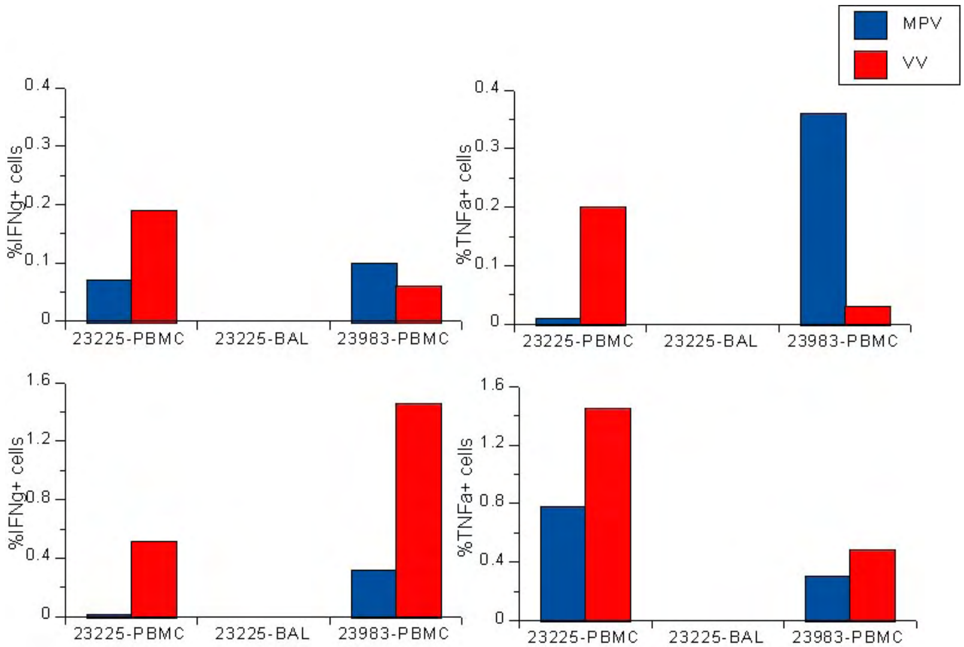


Figure 3 (B) Summary of representative CD4 responses. CD4 T cells responses were lower than CD8 T cells responses, but followed similar hierarchy with CD4 EM responding more vigorously. Interestingly we were not able to detect any CD4 T cells in BAL.

To define T cell responses to A29R and A35L, we purchased overlapping 15 mer peptides to both ORFs and included B6R and M1R. We decided to utilize overlapping peptides rather than generation of vaccinia virus (VV) vectors expressing each of these MPV ORFs, as there is considerable sequence homology with the homologous VV ORFs, and MPV-infected animals possess CD8+ T cell responses to VV (Fig. 4A and B), which would further compound interpretation of the results. Although we could have potentially replaced the VV ORF with the homologous MPV ORF to define T cell responses to the MPV specific ORF, this would have strayed from our originally proposed Task.

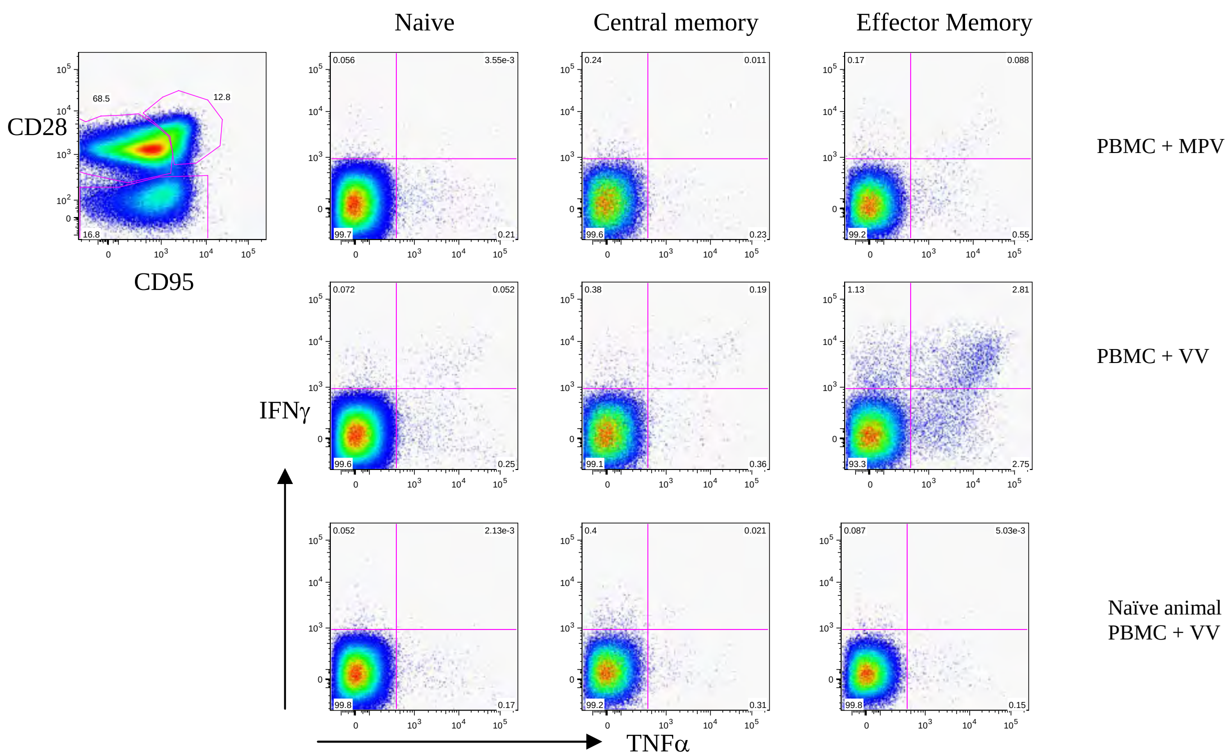


Figure 4A: CD8+ T cell responses to MPV in MPV-infected or naïve animal. PBMC from MPV infected or naive animals were stimulated with either MPV, VV-WR strain as described previously in Hammarlund et al. (1). Frequency of IFNγ and TNFα secreting T cells was determined using intracellular cytokine staining (ICCS) assay. Stimulation of PBMC with VV leads to a strong cytokine response dominated by CD8 T cells. Surprisingly, stimulation of PBMC with MPV did not result in a detectable cytokine response, indicative of immune evasion mechanisms employed by MPV.

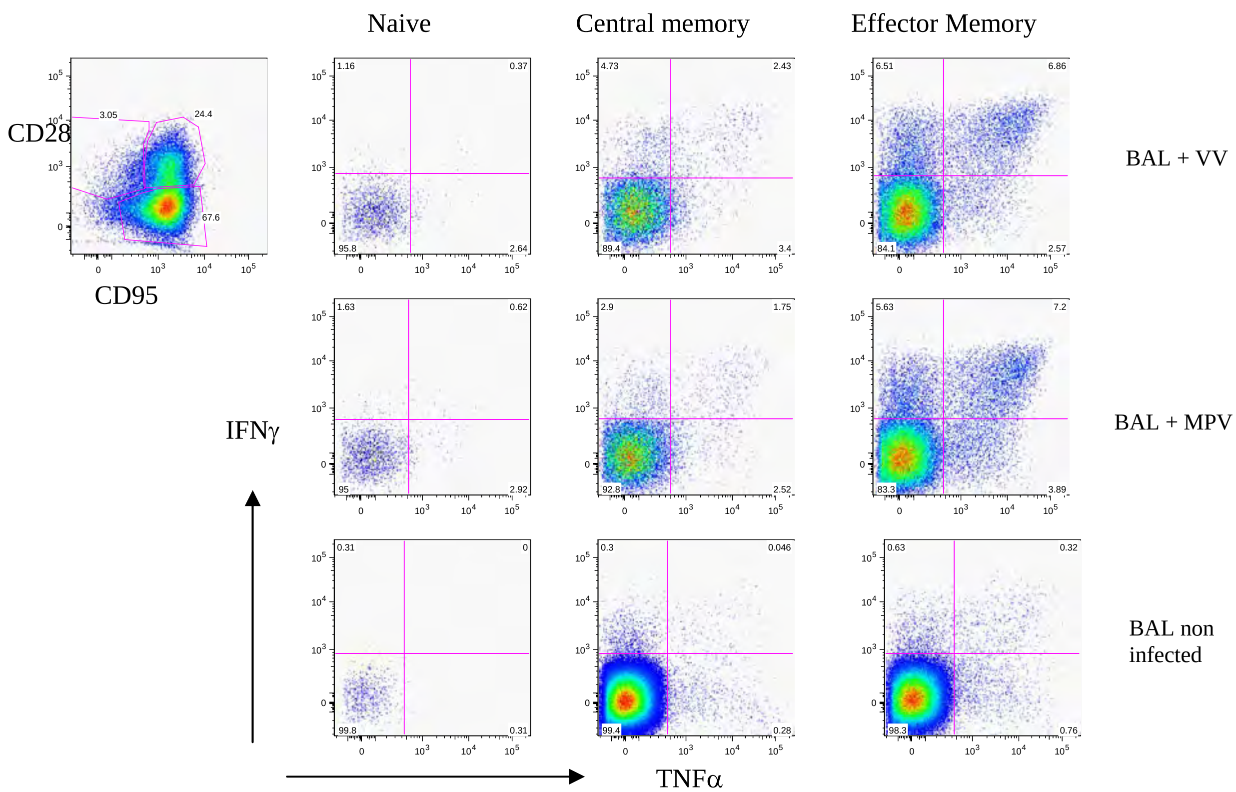


Figure 4B: CD8+ T cell responses to MPV in MPV-infected or naïve animal. Same analysis described in Figure 4A was carried out on T cells isolated from bronchial alveolar lavage (lung wash -BAL). Interestingly, stimulation with both VV and MPV resulted in a strong cytokine response, once again mostly mediated by CD8 T cells. MPV was not able to subvert the CD8 response in this tissue.

We evaluated whether one MPV-infected animal is capable of specifically recognizing MPV A29R, A35L, B6R and M1R antigens in ICCS assays. Here, we utilized bronchial alveolar lavage cells collected at necropsy from one animal to test for T cell responses with MPV peptide pools. Pool 1 contained overlapping peptides for M1R and B6R, pool 2 contains peptides for B6R and A29L, and pool 3 contains peptides for A29L and A35R. As negative control for our ICCS assays, we utilized simian varicella virus (SVV) as this animal had no exposure to SVV and this provides a virus specific negative control to adequately evaluate the general anti-viral response in the lung.

As shown in Figure 5, we were able to effectively detect MPV-specific T cell responses in one animal. Specifically, we found higher levels in the CM CD4+ subset to MPV peptide pool 2 and significantly higher levels of EM CD4+ cells to MPV peptide pools 1 and 3. We observed limited numbers of CM CD8+ subset to MPV pool 2, but rather high levels of EM CD8+ cells to MPV peptide pool 3.

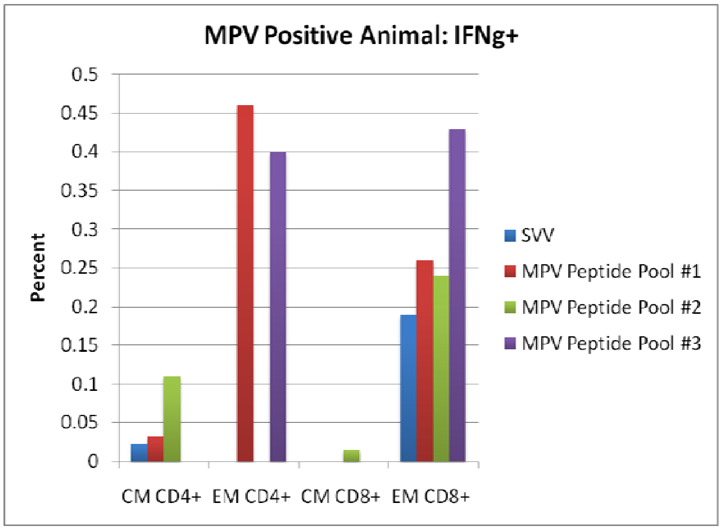


Figure 5: Percent IFNg+ T cell subsets in the BAL of MPV-infected animal. T cells isolated from BAL were analyzed by ICCS for responses to MPV peptide pools and compared to SVV-specific responses.

The higher level of EM CD4+ and EM CD8+ cells to MPV peptide pool 3 was encouraging, as this pool contains the entire overlapping peptides for A35R, which is present in our RhCMV vector (SA1). To evaluate whether our initial RhCMV/A35R vector is capable of stimulating T cell responses to MPV A35R, we vaccinated two RM with the RhCMV/A35R vector by subcutaneous infection and monitored T cells isolated from the BAL at 35 days post-vaccination for responses to MPV peptide pool 3 compared to T cell responses to SVV or MPV peptide pool 1.

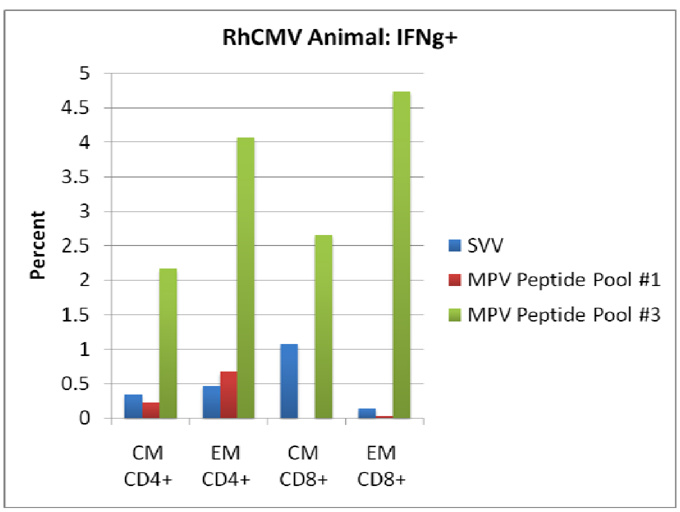


Figure 6: **Percent IFNg+ T cell subsets in the BAL of RhCMV/A35R vaccinated animal.** T cells isolated from BAL were analyzed by ICCS for responses to MPV peptide pools 1 and 3, and compared to SVV-specific responses.

As shown in Figure 6, this one vaccinated animal possesses robust T cell responses that were detected with MPV peptide pool 3. The second animal did not develop as robust T cell responses. We intend to re-vaccinate both animals to the RhCMV/A35R$_{cyto}$ vector to determine if subsequent vaccination with a vector that may express A35R differently is capable of stimulating a stronger T cell response.

SA3B: We will determine the antiviral antibody response mounted against the MPV structural proteins, A29L and A35R, in MPV-infected RM.

We are currently in the process of addressing this sub-aim.

FUTURE WORK:

As we were capable of detecting MPV A35R specific responses in one of the RhCMV/A35R vaccinated animals, we intend to vaccinate the animals with the RhCMV/A35R$_{cyto}$ vector and monitor T cell subset changes. We anticipate that both animals will have enhanced T cell responses as measured by our ICCS assay. After characterization of the response, we intend to challenge both animals with WT MPVZ utilizing the intrabronchial infection model that we developed with this support and monitor the course of disease. If successful protection is observed, we plan to submit an application to evaluate this vector approach in a larger cohort of animals to provide statistical significance.

KEY RESEARCH ACCOMPLISHMENTS:

Construction and in vitro characterization of RhCMV/MPV vaccine vector
Defining pathologic outcome of intrabronchial MPV-inoculation
Defining host response to MPV infection with biotelemetry
Characterization of host immune response to pathogenic MPV infection
In vivo infection of RM with RhCMV/MPV vaccine vector
Detection of MPV A35R specific CD4+ and CD8+ T cell responses in RM infected with RhCMV/MPV vector

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

We have accomplished or are nearing completion of the specific aims proposed in the initial application. In Specific Aim 1, we were successful in creating two RhCMV vectors each containing the MPV A35R gene. Animals have been experimentally inoculated with our first vector and the animals will be re-infected with the second RhCMV vector. Following extensive characterization to assess immunological responses to the MPV antigen, the animals will be challenged. Specific Aim 2 has been completed. We have established the intrabronchial inoculation MPV: RM model and are nearing completion of the studies originally described in Specific Aim 3, which is to characterize the host immune response to MPV A35R and A29L. Taken together, we are now well positioned to define the efficacy of RhCMV/MPV vectors to elicit a protective immune response to MPV challenge in the rhesus macaque animal model.

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